Ecological Diagnostics for Marine Mammals: Appraisal of molecular-based methods for dietary and age estimation

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"*The ballast of factual information, so far from being just about to sink us, is growing less daily. The factual burden of a science varies inversely with its degree of maturity*"

Sir Peter Medawar, OM. "Two conceptions of science",

Henry Tizard Memorial Lecture, Encounter 143, August 1965

Declarations

Originality;

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of the my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

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Ethics;

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

This research was approved by the Animal Ethics Committee of the University of Tasmania

(Permit # A0008315).

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Peer reviewed literature already published from parts of this thesis

Paper 1:

Dunshea, G., N. B. Barros, R. S. Wells, N. J. Gales, M. Hindell, A. and S. N. Jarman (2008) Pseudogenes and DNA-based diet analyses; a cautionary tale from a relatively well sampled predator-prey system. *Bulletin of Entomological Research* 98: 239-248.

Paper 2:

Dunshea, G. (2009). DNA-based diet analysis for any predator. *PLoS One* 4(4): e5252.doi:10.1371/journal.pone.0005252.

Paper 3:

Garde E, A.K. Frie, **G Dunshea** , SH Hansen, KM Kovacs, C Lydersen (2010) Harp seal ageing techniques-teeth, aspartic acid racemization, and telomere sequence analysis. *Journal of Mammalogy*, 91: 1365–1374.

Paper 4:

Dunshea, G., D. Duffield, N. Gales, M. Hindell, R. S. Wells and S. N. Jarman (2011). Telomeres as age markers in vertebrate molecular ecology. *Molecular Ecology Resources,* 11: 225-235.

Statement of Co-Authorship

The following people and institutions contributed to the publication of the work undertaken as part of this thesis:

Paper 1 ***G. J. Dunshea*** *(70%), N. B. Barros (5%), R. S. Wells (5%) N. J. Gales (5%) M. A. Hindell (5%), S. N. Jarman (10%)*

Paper 3 ***G. J. Dunshea*** *(20%), Garde E (35%), A.K. Frie (25%), S.H. Hansen (10%), K.M. Kovacs (5%), C Lydersen (5%)*

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Paper 1

G. J. Dunshea contributed to the idea of the manuscript, its formalisation and development, performed the laboratory work, data collection, data analysis and wrote the paper

S. N. Jarman contributed to the idea of the manuscript, its formalisation, development and manuscript refinement and presentation

N. B. Barros and R. S. Wells assisted with sample collection and manuscript refinement and presentation

N. J. Gales and M. A. Hindell assisted with manuscript refinement and presentation

Paper 3

G. J. Dunshea, contributed to the idea of the paper, provided harp seal telomere assay data, data analysis and interpretation, wrote the telomere part of the paper and assisted with manuscript refinement and presentation

Garde E. contributed to the idea of the paper, its formalisation and development , provided aspactic acid racemization data, its analysis and interpretation and wrote the bulk of the paper

A.K. Frie and S.H. Hansen contributed to the idea of the paper, its formalisation and development , provided teeth growth layer group analysis, sample collection and manuscript refinement and presentation

K.M. Kovacs and C Lydersen contributed to the idea of the paper, its formalisation and development, performed data analysis and manuscript refinement and presentation

Paper 4

G. J. Dunshea, contributed to the ideas in the paper, their formalisation and development, performed most of the laboratory work, data collection, data analysis and wrote the paper

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Abstract

Traditional molecular ecology has focused on describing the historic processes that lead to contemporary patterns of diversity within populations, species and higher taxa. Molecular tools can also identify the origin of biological material and shed light on contemporary ecological processes of populations and individuals. This thesis is concerned with evaluating the efficacy of some of these latter nascent applications for diagnosing near real-time ecological information in marine mammals. The applications under investigation were (i) DNA-based methods to identify the prey of cetaceans and (ii) using the size of telomeres within the life of an individual to estimate the age of individuals in the Pinnipedia and Cetacea.

Diet samples like faeces are complex mixtures of predator, prey and symbiont DNA and as such they require techniques that can exclusively target prey DNA. Previous DNA-based diet studies had employed species- or group-specific polymerase chain reaction (PCR) primers to achieve this and thus were implemented *ad hoc* or required *a priori* diet knowledge, which limited their scope. I developed a prey detection method that employed novel PCR primers widely complementary ('quasi-universal') for most animal 16S mitochondrial DNA (mtDNA) and a restriction enzyme to selectively exclude predator mtDNA. The method requires no *a priori* diet knowledge and can be applied to other predators with a minimum of modification. Faecal samples were collected from two sources; captive bottlenose dolphins (*Tursiops sp.*) fed a known diet and free-ranging bottlenose dolphins from Sarasota Bay, Florida.

Two techniques were applied to detect prey DNA in the captive samples; amplification of a small mtDNA fragment using a species-specific PCR primer pairs designed to detect a known prey species and the 'quasi-universal' method. Using the species-specific method, a prey signal was detected within 4-7 hours of feeding the captive dolphins the known diet and persisted for at least 12-19 hours after the diet ceased. After the first detection, 60 +/- 12% (mean +/- 95% CI) of captive samples contained a prey DNA signal using species-specific methods. The 'quasi-universal' method was applied to 12 samples from within the time period with a known diet of 10 prey species from 3 Phyla. Up to six prey species were detected per sample (range 0-6, mean 3.2 +_ 1.7 (SD) species) and all but one prey species consisting of 2% wet weight of the total diet were detected across all samples. No prey DNA was detected from one captive sample using this method. Estimates of prey item amplicon amounts showed congruence with the total proportion of wet weight of most prey items in the diet, though variability introduced through sampling amplicon clone libraries puts wide confidence intervals on these results.

The 'quasi universal' method was then applied to 15 faecal and 9 gastric samples from 19 free-ranging Sarasota Bay dolphins. Thirty two prey molecular operational taxonomic units (MOTUs) were identified across all samples (range 0-9, mean 3.7 +/- 2.2 per individual), consisting of 28 taxonomic assignments, 18 of which were species level identifications. One

sample did not contain prey DNA. The difference in results between samples from the captive animals and free-ranging animals suggest that factors such as sample collection methods, sample amount, sample storage duration and whether animals consume live or dead prey may affect the efficacy of DNA-based techniques, which has ramifications for interpreting results from captive feeding trials. These results were also congruent with diet data from this population via traditional hard-parts analysis of stomach contents from stranded individuals.

An unexpected consequence of using restriction enzymes to exclude predator mtDNA was the appearance of nuclear mitochondrial pseudogenes (NUMTs) in samples. The appearance of NUMTs in 15 faecal samples from Sarasota Bay dolphins was further investigated, in order to understand their impact on DNA-based dietary analysis in a field situation. Nine unique NUMT paralogs detected in 13 of 15 samples were represented by 1-5 paralogs per sample and were estimated to be between 5-100% of all amplicons produced per sample. The diversity of prey DNA and the proportion of NUMT amplicons per sample were related to real-time PCR cycling characteristics, with lower prey diversity and a higher proportion of NUMTs recovered with increasing real-time PCR threshold cycle values. This indicated that low DNA yields from diet samples are more likely to have NUMTs detected and less likely to contain prey DNA using this technique. This predator-prey system is relatively well sampled, which facilitated ease of identification of NUMTs, however for many study systems this may not be the case.

Telomeres are nucleoprotein structures on the end of eukaryote chromosomes that consist of regions containing 'telomere-like' and 'true' telomere tandomly repeated DNA sequence and single stranded telomere sequence overhangs on the 3'end of each anti-parallel DNA molecule, each with associated proteins. They change size throughout the life of many animals, suggesting that they be a molecular means to estimate animal age. To examine whether telomeres would be useful to estimate the age of pinnipeds and cetaceans, samples were collected from populations of three model species where the age of individuals was known or could be relatively inferred; harp seals (*Pagophilus groenlandicus*), bottlenose dolphins and southern right whales (*Eubalaena australis*).

In Harp Seals, telomeres were measured using two techniques, (i) de-naturing terminal restriction fragment analysis and (ii) quantitative PCR (Q-PCR). There was no relationship between age and telomere length using either telomere measurement method, however there was a strong correlation between the methods, indicating that they were comparable. Telomere dynamics in cetaceans were then investigated. Previous studies had shown that satellite DNA in Mysticete cetaceans contains telomere sequence repeats that may bias telomere measurement techniques. This was investigated in Odontocete cetaceans by characterizing interstitial telomere sequence (ITS – that is, telomere repeat DNA sequence that is not a part of true telomeres) in Bottlenose Dolphins. It was found that substantial ITS exists in bottlenose dolphins, and given its presence in closely related Mysticete cetaceans, most likely all cetaceans. The presence of this ITS made denaturing TRF analysis difficult to

interpret and so attempts were made to measure Bottlenose Dolphin telomeres using non-denaturing TRF assays and Q-PCR assays. Attempts at non-denaturing assays were unsuccessful and further efforts focused on Q-PCR assays. No relationship was found between Q-PCR telomere metrics and age in bottlenose dolphins. In four instances longitudinal samples were available from the same bottlenose dolphin individual a number of years apart and these were compared. While two samples showed the typical pattern of decline in telomere size with age, one showed no discernable change with age and one individual displayed an apparent gain in telomere sequence with age.

Given these results a small subsection of 6 cow-calf pair Southern Right Whale samples were initially analysed. Four adults appeared to contain shorter telomere sequences than their calves although in two cases, calves contained less telomere sequence then their presumed mother. In light of these results the use of telomeres to estimate age of individual marine mammals did not appear a valid technique and could not be recommended.

Overall, this study achieved its aims of appraising the efficacy of these nascent molecular ecology techniques in marine mammals. In the first instance, DNA-based diet analysis appears to hold great promise for analysis of cetacean diet. The methods were sensitive, identified prey to the lowest taxonomic level in many cases, and made use of samples that cannot be used for traditional diet analyses. They allow high-resolution prey detection of live animals, a feat that cannot be otherwise achieved in cetaceans failing direct observation of feeding events. Additionally the 'quasi-universal' method can be applied to any predator where its 16S mtDNA sequence is known or can be gained, and the biases of the technique can be inferred by using current data from databases such as GenBank. Conversely, telomeres appear to hold little use for age estimation in marine mammals. All methodological issues aside, there are many exogenous and endogenous influences other than chronological time on individual telomere dynamics and these are not well understood. It is not recommended that telomeres be used for age estimation in any animal group without considerably more work. The latter outcome is as useful as the former, since any emerging technique (no matter how promising) must be put through rigorous critical appraisal in order to understand whether the application is warranted at all, or if so what the caveats might be.